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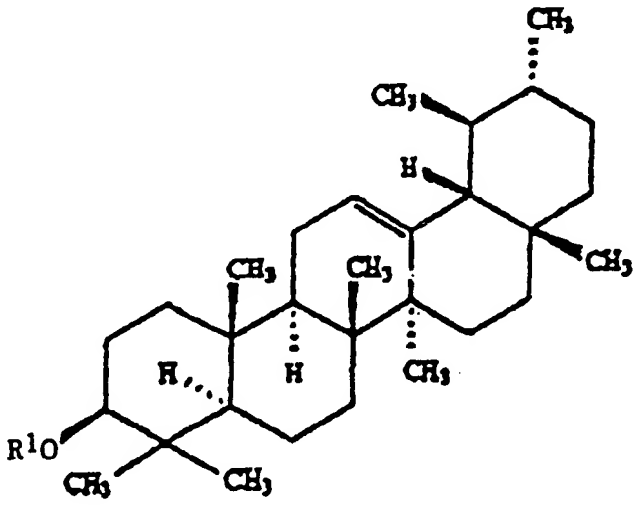

(II)

(57) Abstract

A method of antiinflammatory, and more particulaly antiarthritic, treatment of a human or animal patient comprises administering to the patient an effective amount of a compound of general formula (II) in substantially pure form, wherein R^1 represents hydrogen or an acyl group derived from a long chain fatty acid, preferably a fatty acid having (10) or more carbon atoms. Particularly preferred compounds are α-amyrin and α-amyrin palmitate.

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TRITERPENE COMPOUNDS HAVING ANTIINFLAMMATORY ACTIVITY.

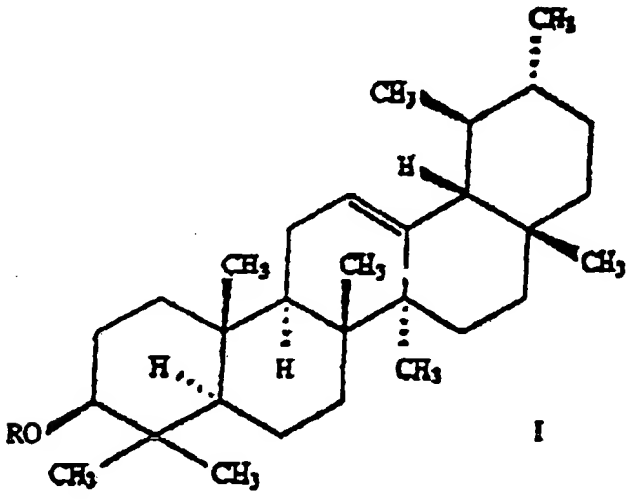
This invention relates to triterpene compounds having antiinflammatory activity. More specifically, this invention relates to novel derivatives of the triterpene, α-amyrin, which have been found to have useful antiinflammatory activity, particularly antiarthritic activity, and to the use of α-amyrin itself and of these novel derivatives in antiinflammatory and more especially antiarthritic treatment of human or animal patients.

A water or alcoholic extract of *Alstonia boonei* root barks, *Rauvolfia vomitoria* root barks and *Elaies guineensis* nuts without pericarp is used as a herbal cure for arthritis in Ghana, and previous studies have shown that the extract was effective in reducing swelling in acute and chronic models of inflammation (Kweifio-Okai, 1991a and b). *Alstonia boonei* is the major plant constituent of the herbal preparation responsible for the antiarthritic effect (Dalziel, 1937) and some of the triterpene constituents of the root barks (Faparusi and Bassir, 1972) have been shown to be antiinflammatory, antiarthritic and antiproteolytic (Gupta *et.al*, 1969 and 1971; Chaudhari *et.al*, 1974; Chaturvedi *et.al*, 1974). For those reasons, the triterpenes (α-amyrin acetate, β-amyrin acetate, β-amyrin and lupeol acetate) isolated from a petroleum ether extract of *Alstonia boonei* root barks were tested for their antiarthritic effect in adjuvant rats (Kweifio-Okai and Carroll, 1992). The results demonstrated antiarthritic effects but with varying degrees of toxicity as shown by swellings of the liver, kidney and spleen.

It has now been found that a particular class of α-amyrin derivatives which are not present as such in any of the plants in the herbal preparation, but which

may be present in the combination of plants in the herbal preparation, when prepared and used in substantially pure form have useful antiinflammatory, and more especially antiarthritic activity. In the work leading to the present invention, complete Freund's adjuvant (CFA)-induced arthritis was used as the animal model for human arthritis. As in human arthritis, a granulomatous outgrowth of synovial tissue called pannus invades and destroys joints in adjuvant rats. In rats, pannus formation occurs 5-10 days after adjuvant inoculation (Pearson and Wood, 1963), and therefore testing was performed from days 11 to 19 postadjuvant, i.e. therapeutically rather than prior to or during the induction of arthritis. It was considered that this mode of testing was more practical and would eliminate drugs which could well inhibit arthritis in the developmental stages but without effect on established arthritis (Glenn *et.al*, 1977).

According to the present invention, there are provided compounds of the general formula I:



I

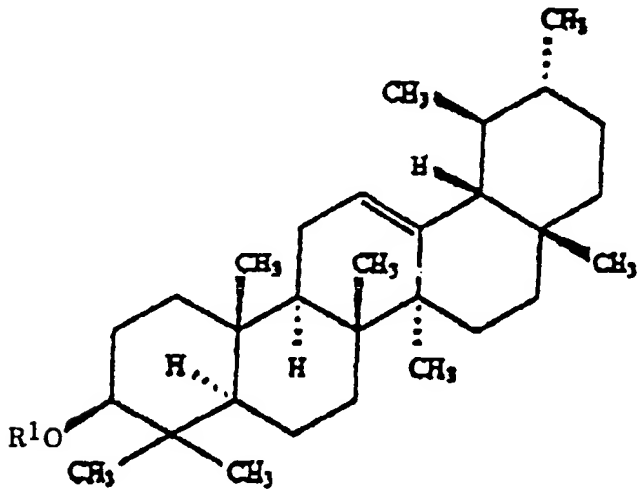
wherein R represents an acyl group derived from a long chain fatty acid.

Preferably, the compounds of general formula I are provided in substantially pure form. By "substantially pure" is meant at least 75%, preferably at least 85%, and more preferably at least 95%, pure.

Suitably, the acyl group is derived from a fatty acid having 10 or more carbon atoms. Such fatty acids include both saturated and unsaturated acids, including for example capric, lauric, myristic, palmitic, stearic, and arachidonic acids as saturated acids, and myristoleic, palmitoleic, oleic, linoleic, linolenic and arachiodonic acids as unsaturated acids. The particularly preferred acyl group of the invention is derived from palmitic acid.

The compounds of general formula I above may be produced synthetically from α-amyrin acetate (Kweifio-Okai and Carroll, 1992) by hydrolysing the acetate to α-amyrin, followed by esterification with the appropriate long chain fatty acid, or a derivative such as an acid halide of the fatty acid, for example palmitic acid or palmitoyl chloride.

Furthermore, in another aspect of this invention extends to a method of antiinflammatory and more especially antiarthritic treatment of a human or animal patient, which comprises administering to said patient an effective amount of a compound of the general formula II in substantially pure form:



II

wherein R^1 represents hydrogen or an acyl group derived from a long chain fatty acid, preferably a fatty acid having 10 or more carbon atoms as described above.

The invention further extends to the use of a compound of the general formula II in substantially pure form in the manufacture of a medicament for antiinflammatory and more especially antiarthritic use.

The present invention also provides a pharmaceutical composition for antiinflammatory and more especially antiarthritic use, comprising a compound of the general formula II in substantially pure form, together with a pharmaceutically acceptable carrier or diluent.

The preferred compound of the present invention, α-amyrin palmitate, has been shown to reduce ankle swelling caused by the subplantar injection of complete Freund's adjuvant. The drug was given orally daily at 56mg/kg body weight from days 11 to 19 postadjuvant and therefore the effect observed was that on established arthritis. The reduction in ankle swelling was about 32% whether the ankle diameters were compared after 6 days of administration or over the entire period of administration using regression analyses. Pannus would have been present by day 11 of adjuvant (Pearson and Wood, 1963) after the compound was administered, after which the compound caused no further increases in ankle diameter. There is histological evidence that this compound prevented further pannus growth, infiltration and destruction of the joints in adjuvant rats.

Initial confirmation of the antiarthritic effects of α-amyrin palmitate came from measurements of serum hyaluronate. Since it was found that serum hyaluronate increases in active human rheumatoid arthritis (Engstrom-Laurent and Hallgren, 1985) and in lactobacillus casei, mycobacterium adjuvant and collagen 11 models of arthritis in rats (Goldberg and Rubin, 1989; Smedegard *et.al.*, 1989) serum hyaluronate has been accepted as a reliable marker of the severity of adjuvant arthritis in rats and in screening potential antiarthritic agents. In this study, the preferred compound significantly reduced adjuvant levels of serum hyaluronate. Serum hyaluronate levels therefore confirm the ameliorative effects of this compound on adjuvant swelling.

α-amyrin palmitate, initially shown to be antiarthritic at the acute stage of adjuvant arthritis (days 11-19), has subsequently been shown to be similarly antiarthritic at the chronic stage of adjuvant arthritis (days 32-50). As at the acute stage, α-amyrin palmitate at the chronic stage returned the increases in serum hyaluronate and circulating granulocytes towards non-arthritic levels and corrected the anaemia of adjuvant arthritis. At the chronic stage, arthritic joint histopathology was improved by the oral administration of α-amyrin palmitate. This is in agreement with the effect at the acute stage. At both stages, reduced cellular infiltration of bone marrow synovium and synovial cavity and periarticular tissue were observed. There was also reduced cartilage destruction. At the chronic stage,α-amyrin palmitate appeared to increase new periosteal bone formation. In addition, chronic arthritic rats treated with α-amyrin palmitate showed reduced uptake of radioactive IgG, known to be a useful marker of inflammatory arthritis.

It has also been shown that α-amyrin and lipid esters of α-amyrin are specific inhibitors of 5-HETE synthesis. Coupled with the knowledge that 5-HETE levels increase in the synovial fluid and tissue of patients with rheumatoid arthritis and spondyloarthritis, this finding also suggests that these compounds may be beneficial in antiarthritic therapy. The demonstration of inhibition of 5-lipoxygenase activity in human neutrophils is unique in antiarthritic activity - there is no antiarthritic drug in current usage which relies on the inhibition of lipoxygenase activity for antiarthritic effect. Drugs that inhibit only cycloxygenase products of arachidonic acid (the prostaglandins) are limited in their effects because the more potent pro-inflammatory substances are found in lipoxygenase pathways.

Antiarthritic agents are often limited by their deleterious effects (Bonta *et.al.*, 1980). In the present study, liver, kidney and spleen weight changes were followed together with circulating RBC levels to identify possible toxicity of the preferred compound for future studies. The observations that liver and kidney weights did not increase in the presence of this compound would suggest an

absence of toxicity. However Barritt and Whitehouse (1977) have shown that liver weight could remain unchanged in the presence of functional impairment. Spleen weight increase may primarily account for the moderate anaemia of adjuvant arthritis because the anaemia was normocytic in the absence of liver and kidney weight increases. The basis for the correction of anaemia by this compound is unclear but it is important to note that the compound halved the spleen weight increase in adjuvant rats. More detailed study is required to elicit any latent toxicity of the preferred compound but the results obtained here, compared with the toxic manifestations of triterpenes from *Alstonia boonei* only (Kweifio-Okai and Carroll, 1992), confirm the rationale behind the combination of plants in the herbal preparation used in antiarthritic therapy in Ghana. In this combination, *Alstonia boonei* is responsible for the antiarthritic effect, *Rauvolfia vomitoria* provides sedative cover and *Elaies guineensis* reduces toxicity (Kweifio-Okai, 1991a). *Elaies guineensis* could reduce toxicity by rendering a triterpene of *Alstonia boonei* as prodrug to limit gut destruction or in a form that enables gut absorption to bypass the usual metabolic routes (Van Arman, 1976).

Further details of the present invention, and in particular of the production and activity of the preferred compound α-amyrin palmitate, are set out in the following Examples, and in the accompanying Figure, which shows:

Figure 1 Percent change in right ankle diameter after 11 days of adjuvant (CFA) and in the presence of the triterpene lipid ester, α-amyrin palmitate. Significance of difference of slope from adjuvant control: *$P < 0.05$; **$P < 0.001$.

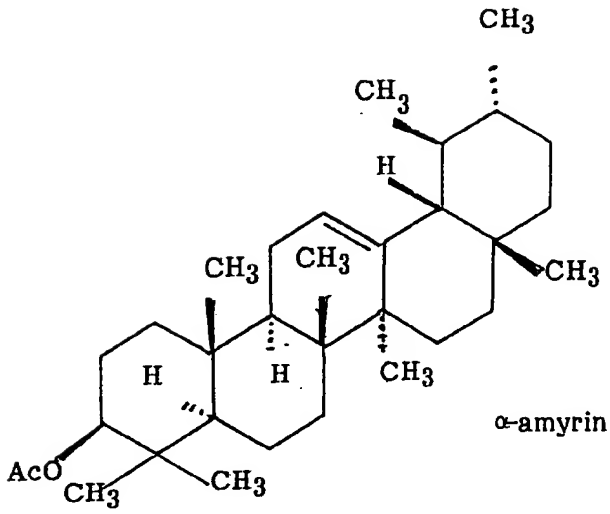
EXAMPLE 1

Preparation of α-amyrin palmitate

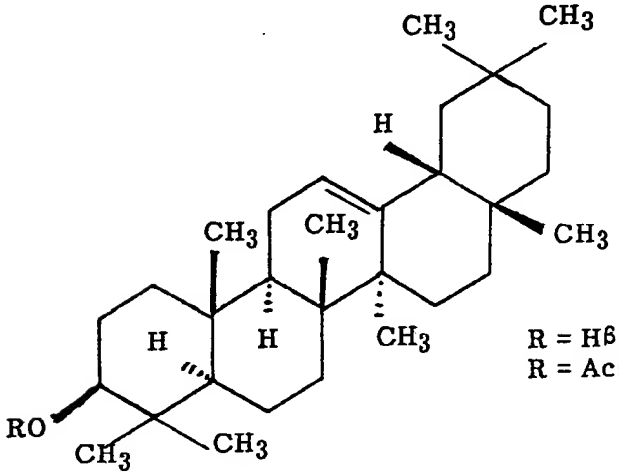
(i) Triterpene isolation from *Alstonia boonei* root barks.

The triterpenes, α-amyrin acetate, β-amyrin acetate, β-amyrin and lupeol acetate were isolated from the petroleum ether extract of *Alstonia*

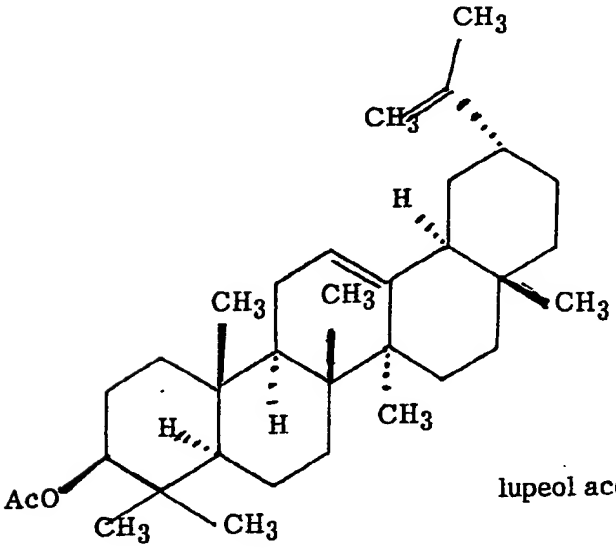
boonei de Wild root bark. The crude petroleum ether extract (8g) obtained from a batch of powdered root bark (300g) was separated by vacuum liquid chromatography on silica using petroleum ether/ethyl acetate (9:1). The terpene acetate fraction (4.5g) was further purified by reverse phase h.p.l.c. {elution with dichloromethane/acetonitrile (30:70)} to yield lupeol acetate (438mg), α-amyrin acetate (1260mg) and β-amyrin acetate (200mg). The more polar terpene alcohol fraction (1g) from the initial vacuum liquid chromatography was recrystallised to yield pure β-amyrin (990mg). From a proton NMR spectrum of the crude petroleum ether extract it was estimated that the root bark contains 1.116% α-amyrin acetate, 0.186% β-amyrin acetate, 0.254% β-amyrin and 0.186% lupeol acetate.



α-amyrin acetate



R = Hβ=amyrin
R = Acβ-amyrin acetate



lupeol acetate

(ii) Preparation of α-amyrin palmitate.

α-amyrin acetate isolated from *Alstonia boonei* root barks as described above was hydrolysed with ethanolic sodium hydroxide to α-amyrin, and the α-amyrin esterified with palmitoyl chloride to α-amyrin palmitate, according to the reaction scheme:

1. α-amyrin acetate $\xrightarrow{\text{heat in NaOH/ethanol}}$ α-amyrin

2. α-amyrin $\xrightarrow[\text{Dichloromethane}]{CH_3(CH_2)_{14}\,\overset{O}{\overset{\|}{C}}Cl}$ α-amyrin palmitate

EXAMPLE 2

Effect of α-amyrin palmitate on acute adjuvant arthritis.

A. Materials and Methods

Animal stock.

Five-and-a-half-week-old outbred Wistar rats weighting between 107 to 130g (Monash University Animal House, Clayton, Australia) were used. Animals were kept in groups of 5 at 23 °C on a 12:12hr light/dark cycle and had free access to food and water throughout the experiment.

Induction of arthritis.

10 rats were inoculated with complete Freund's adjuvant (CFA) containing additional 10mg/ml of *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI). Each rat received a subplantar injection of 150µl of CFA in the midline mid-metatarsal region of the right hindfoot pad. Five control rats received an equivalent amount of saline. From days 11 to 19, 5 adjuvant rats were fed orally daily with the triterpene, α-amyrin palmitate (also referred to herein as CK091 (56mg/Kg body weight in 1ml of drinking water). 5 control rats and the other 5 adjuvant rats received equivalent amounts of drinking water each time.

Anteroposterior diameters of the ankles on injected and contralateral limbs were measured with sliding vernier scale on days 0 and 11 and thereafter every 2 days through to day 19. On day 19 blood was removed by cardiac puncture under ether anaesthesia at two stages. Firstly 1 ml syringe containing heparinized beads and 18" gauge needle was used to withdraw 500-750µl of blood for measurement of blood cells by routine techniques. Then an additional 2 to 3 ml of blood was withdrawn with a 5ml syringe (non-heparanised), allowed to clot and the serum separated by centrifugation. 100µl of serum was used for duplicate measurements of serum hyaluronate (Pharmacia HA Test 50, Pharmacia Diagnostics AB, Uppsala, Sweden). The liver, spleen and kidneys were removed for weighing and right and left limbs were cut above the ankles for histological study. Data was analysed by multiple t-test in which unpaired Student's t-test was used to compare the drug group with either the single adjuvant control group or the single non-adjuvant control group (Sinclair, 1988). Linear regression analysis by the least square fit was used to assess significant correlation of ankle swelling against time. Significance of difference between regression coefficients (slopes) was also assessed (Petrie, 1987). A $P<0.05$ was considered significant for the above tests.

B. Results

Ankle diameter changes and serum hyaluronate.

Control rats did not show any significant changes in injected (ipsilateral) limb ankle diameters during the 19 days of the experiment (Table 1). Similarly there were no changes in contralateral ankle diameters. Adjuvant increased ipsilateral ankle diameters to 94% of preinjection values by day 19 but there was no transfer of adjuvant swelling to the contralateral ankle. The use of older Wistar rats ensured a stable ankle diameter from the beginning of this experiment and a greater response to adjuvant at the ipsilateral limb but as previously (Kweifio-Okai, 1991b) contralateral limb was not nearly as responsive. By day 11, ankle swelling was statistically similar in the two adjuvant groups, 67±6% in the adjuvant control group and 50±6% in the group which subsequently received the triterpene (Table 1). Histological sections would show that in the range of

adjuvant swellings observed in the 2 groups, there was pannus formation and the infiltration of joint (Kweifio-Okai and Bird, unpublished observations). In Table 1 CK091 reduced adjuvant swelling by 20% after 2 days of administration and then after 6 days by an average of 32%. Regression analyses of the rate of ankle diameter change from days 11 to 19 postadjuvant (Fig.1) showed that only in the absence of CK091 was statistical significance of change observed ($P < 0.001$) and the slopes were significantly different ($P < 0.05$). From the regression analyses, ankle diameter increased by 34% in adjuvant rats and by 3% in the presence of CK091 - reduction of 31% by CK091.

In Table 2, adjuvant caused an apparent (+58%) but non-significant increase ($P > 0.1$) in serum hyaluronate. CK091 significantly reduced serum hyaluronate of adjuvant rats by 48% ($P < 0.01$).

White blood cell count.

In Table 3, granulocytes and monocytes increased significantly in adjuvant rats but the increase in lymphocytes did not reach statistical significance. The apparent increase in total WBC in adjuvant rats (+14,700/mm^3) was achieved by a greater increase in granulocytes (+8535, df = 8, $P < 0.05$) than agranulocytes (+6165, N/S). In the presence of CK091 white blood cells were lower than adjuvant levels but were neither significantly different from them, nor from non-adjuvant control levels. Also in Table 3, total neutrophils and the segmented types as percent of total WBC count increased significantly in adjuvant rats but the increases in the presence of CK091 did not reach statistical significance. Percent banded neutrophils, the less mature neutrophils, remained unchanged in adjuvant rats with and without CK091.

Organ weight changes and red blood cell counts.

In Table 4, neither adjuvant nor CK091 had any effect on liver and kidney weights. Spleen weight increased in adjuvant rats in the presence (+46%) and absence (+89%) of CK091. Adjuvant significantly reduced total RBC count (-

10%), but RBC volume was unchanged. CK091 returned RBC count to non-adjuvant control levels (Table 4).

TABLE 1

RIGHT ANKLE DIAMETER CHANGES IN CONTROL AND ADJUVANT RATS WITH AND WITHOUT TRITERPENE

Tabular values represent mean ± S.E.M. changes from 0 time readings, N=5/group.

Treatment	O Time ankle diameter (mm)	Ankle diameter %				
		+11	+13	+15	+17	+19 days
(a) Control	6.57 ± 0.07	6 ± 2	10 ± 3	11 ± 2	7 ± 2	9 ± 3
(b) CFA only	6.25 ± 0.16	67 ± 6	81 ± 6	81 ± 5	88 ± 8	94 ± 7
(c) CFA + CK091	6.57 ± 0.12	50 ± 6	61 ± 5	64 ± 7	53 ± 9	66 ± 7
(b-a)	-0.32	+61^{xx}	+71^{xx}	+70^{xx}	+81^{xx}	+85^{xx}
(c-b)	+0.32	-17	-20^{x}	-17	-35^{x}	-28^{x}

Statistically significant for comparison specified: $^{x}P<0.05$, $^{xx}P<0.001$

CKO91, a triterpene lipid ester present in *Alstonia boonei*, *Rauvolfia vomitoria* and *Elaies guineensis*, was given orally daily (56mg/Kg wt) from days 11 to 19 postadjuvant.

TABLE 2

SERUM HYALURONATE IN CONTROL AND ADJUVANT RATS WITH AND WITHOUT TRITERPENE

Data at day 19 of adjuvant, 8 days after administration of triterpene.

Treatment	Mean serum hyaluronate ± S.E.M. (µg/l)	N	P
Control	181 ± 34	5	>0.10
CFA only	287 ± 49	4	-
CFA + CK091	149 ± 35	5	<0.01

Comparison with adjuvant control.

TABLE 3

DIFFERENTIAL WBC AND NEUTROPHIL COUNT IN CONTROL AND ADJUVANT RATS WITH AND WITHOUT TRITERPENE

TABULAR VALUES REPRESENT MEAN ± S.E.M. Data at day 19 of adjuvant, 8 days after administration of triterpene. N=5/group except data for CKO91 =4

	CONTROL	CFA	CFA + CKO91
Total WBC/mm^3	9820 ± 589	24520 ± 6367	18325 ± 4006
Granulocytes/mm^3	1292 ± 280	9827 ± 3429^x	5861 ± 2097
Lymphocytes/mm^3	7477 ± 775	10725 ± 3690	10317 ± 1255
Monocytes/mm^3	1051 ± 85	3968 ± 1148^x	2147 ± 1002
Total neutrophils (%WBC)	12.8 ± 3.6	36.2 ± 8.3^x	26.5 ± 6.6
Segmented neutrophils (%WBC)	8.8 ± 2.7	32.0 ± 7.3^{xx}	22.5 ± 5.6
Banded neutrophils (%WBC)	4.0 ± 1.2	4.2 ± 1.2	4.0 ± 1.1

Statistically significant for comparison with non-adjuvant control: $^xP<0.05$, $^{xx}P<0.02$.

TABLE 4

ORGAN WET WEIGHTS AND RBC IN CONTROL AND ADJUVANT RATS WITH AND WITHOUT TRITERPENE

Tabular values represent mean ± S.E.M. Data at day 19 of adjuvant, 8 days after administration of triterpene

	CONTROL	CFA		CFA + CKO91	
	Mean + S.E.M.	Mean + S.E.M.	Change (%)	Mean ± S.E.M.	Change (%)
Liver (g%)	3.87 ± 0.08	3.71 ± 0.18	- 4	3.75 ± 0.09	- 3
Spleen (g%)	0.28 ± 0.01	0.53 ± 0.06	+89^{xx}	0.41 ± 0.04	+46^{xx}
Kidney (g%)	0.80 ± 0.01	0.84 ± 0.04	+ 5	0.87 ± 0.04	+ 9
RBC (10^6/mm^3)	7.16 ± 0.10	6.47 ± 0.26	-10^{x}	7.02 ± 0.06	- 2
MCV (fl)	57.6 ± 1.0	54.9 ± 1.3	- 5	57.9 ± 0.6	0

Statistically significant for comparison with non-adjuvant control: $^{x}P<0.05$, $^{xx}P<0.01$.

N=5/group except RBC data for CKO91 =4.

EXAMPLE 3

Effect of α-amyrin palmitate on chronic adjuvant arthritis

A. Materials and Methods.

Rats and treatment.

Fifty 8-9 week old outbred male Wistar rats weighing between 150-215g (Monash University Animal Centre, Clayton, Australia) were kept at room temperature of 23 °C on a 12:12 light/dark cycle and had free access to water and food (KMM mouse and rat ration, Pakenham, Australia). 34 rats were inoculated with 150 µl of complete Freund's adjuvant containing additional 10mg/ml of *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI) (right hindfoot subplantar). 32 days later, those animals with a satisfactory level of arthritis (n = 24) were randomly assigned to cages in groups of 4. The criteria for successful induction of arthritis (Jacka *et al.*, 1983) were: (i) an ipsilateral (injected) paw diameter increase of 3.4 mm minimum and ipsilateral ankle diameter increase of 3.2 mm minimum by day 11 of adjuvant maintained up to day 32, (ii) severe restriction of movement in affected limbs and pain responses when handled. The arthritic animals received 66 mg/kg body weight of α-amyrin palmitate, synthesised as described in Example 1, or sham fed by oral gavage for 5 days, 48 hours apart, from days 32 to 40 postadjuvant. Body weights, anteroposterior diameters of ankles and dorsoventral heights of food pads were measured on days 0, 11, 18, 32, 34, 36, 38, 40, 42, 46 and 50.

On day 50, 8 each of non-arthritic, treated and untreated arthritic rats were sacrificed by ether anaesthesia, blood removed by cardiac puncture before death and analysed for serum calcium, phosphate, alkaline phosphate, SGOT, SGPT, total bilirubin, glucose, urea and creatinine. Biochemical measurements were made with the KODAK Ektachem DT analyser system using the appropriate slides (Eastman Kodak Company, NY). Blood removed in heparinised syringes into heparinised tubes were analysed for blood cells using routine techniques. Serum hyaluronate was measured in duplicate using the Pharmacia HA 50 test kit

(Pharmacia Diagnostics, AB). The liver, kidney and spleen were removed for weighing. Data was analysed using the unpaired student t-test and a $p<0.05$ was considered significant.

B. Results and Discussion.

Ipsilateral and contralateral ankle and paw diameters of control rats remained unchanged throughout the experiment. Ipsilateral ankle and paw diameters in adjuvant rats significantly increased and peaked on day 11 after adjuvant inoculation (+54% and +113% respectively) and the increases were maintained up to day 50. α-amyrin palmitate made no difference to ipsilateral ankle and paw diameter changes in adjuvant rats during and after administration up to day 50. Contralateral ankle and paw diameters remained unchanged in adjuvant rats with and without the drug. Body weight increases in normal control, treated and untreated arthritic rats were identical over the duration of the experiment.

In Table 5, both treated and untreated arthritic rats had identical serum alkaline phosphatase and Ca^{++}. However serum inorganic phosphate was 17% greater in treated arthritic rats than in control rats and 11% greater than in untreated arthritic rats. Serum hyaluronate increased by 77% in untreated arthritic rats but the 47% increase in treated rats did not reach statistical significance.

In Table 6, granulocytes were the only white blood cells which increased significantly in untreated arthritic rats. The increase was 242% compared with an increase of 171% in treated rats. The latter increase however, was barely significant ($df = 14$; $p<0.10$). Table 6 also shows that neutrophils and the mature (segmented) and immature (banded) types expressed as percent total WBC count increased in both treated and untreated arthritic rats, but treated rats showed a greater increase in immature neutrophils and a lesser increase in mature neutrophils than untreated rats. Anaemia (-5%) of the microcytic (-4%) and hypochromic type was present in untreated arthritic rats (Table 6). In treated rats

RBC count and haemotocrit were normal but the RBCs remained microcytic and hypochromic. Reticulocyte count would suggest that bone marrow production was stepped up significantly in treated rats (+49%) to avert anaemia but not in untreated rats (+27%).

Table 7 shows that by day 50 of adjuvant arthritis, liver and kidney function were preserved both in the treated and untreated arthritic rats. Serum urea was however reduced by 14% in both treated and untreated arthritic rats but only the decrease in treated rats reached statistical significance. Spleen weight increased in both treated and untreated arthritic rats to about the same extent (30-35%).

Various parameters have been used to assess the potential of α-amyrin palmitate as an antiarthritic drug. Physical measurements of local ankle and paw diameters after 32 days of adjuvant inoculation failed to reflect the increasing severity of adjuvant arthritis or its curtailment by α-amyrin palmitate. This is in contrast to previous studies over 17 or 19 days of adjuvant arthritis (see Example 2). During the period of drug administration in the present study, there would be significant joint destruction and some fibrous ankylosis (Pearson and Wood, 1963) and at this chronic inflammatory stage local limb sizes indicate nothing about joint activities.

Biochemical indices of bone metabolism suggested reduced joint inflammation and osteoclastic activity in treated arthritic rats. It has been shown that blood leucocytes, agents for adjuvant joint destruction, and serum hyaluronate which result from articular cartilage degradation increase in clinical and experimental arthritis and are reduced by antiarthritic agents. In the present experiment significant joint inflammation or destruction is implied by the 77% increase in serum hyaluronate on day 50 postadjuvant. Treated rats however, showed reduced and insignificant increases in serum hyaluronate. Blood granulocytes also increased in arthritic rats but the increase in treated rats was not statistically significant. Glenn and others (1977) suggested that drugs inhibited arthritis primarily by reduction of granulocytes even if agranulocytes

were reduced as well. Circulating agranulocytes (lymphocytes) form a greater proportion of white blood cells in the rat than in humans and therefore response to infection in the rat is primarily by increase in circulating granulocytes (Glenn *et al.*, 1977; Van Arman, 1976). The reduced joint inflammatory activity is consistent with observations of reduced uptake of radioactive labelled IgG at the joints of α-amyrin palmitate-treated arthritic rats (Griffiths *et al.*, 1992). The present data would also suggest that joint repair took place in treated arthritic rats - the 17% increase in serum phosphate levels would favour Ca^{++} deposition in bone. The hyperphosphataemia of treated arthritic rats cannot be attributed to haemolysis or destruction of RBC *in vivo* or *in vitro* (normal bilirubin and RBC numbers) not to phosphate leaching from bone (normal Ca^{++}) nor to phosphate retention in renal impairment (normal glucose and creatinine). Nor could it be attributed to metabolic effects since the changes in serum Ca^{++} and PO_4^{--} were inconsistent with the known effects of the major calciotropic hormones - parathormone, calcitonin and active vitamin D_3. Alkaline phosphatase, an index of osteoblastic activity in bone disease in the absence of hepatobiliary disease, did not increase in treated rats. However, it has been suggested that increased osteoblastic activity at local bone sites may not necessarily translate into increases in serum alkaline phosphatase levels.

The results suggest that α-amyrin palmitate is less toxic compared with α-amyrin acetate in previous studies. α-amyrin acetate increased kidney weight by 20%, SGOT by 67% and had no effect on the anaemia of adjuvant arthritis (Kweifio-Okai and Carroll, 1992). In the present experiments, α-amyrin palmitate had no effect on kidney weight and the serum liver enzymes SGOT and SGPT and corrected the anaemia of adjuvant arthritis.

TABLE 5 Biochemical indices of bone turnover in adjuvant arthritic rats treated with α-amyrin palmitate.

	Control	Untreated arthritic	Treated arthritic
Total Ca^{++}(mmol/L)	2.61 ± 0.03	2.54 ± 0.02	2.63 ± 0.05
Inorganic $PO_4^{=}$(mmol/L)	2.24 ± 0.06	2.35 ± 0.07	2.61 ± 0.08^{xxx}
Alkaline phosphatase (U/L)	274 ± 10	266 ± 22	282 ± 22
Serum hyaluronate (ug/L)	100 ± 4(6)	177 ± 22^{xxx}	147 ± 26(6)

Values are mean ± SEM of 8 rats except where indicated in parenthesis. Significance of difference from normal rats was calculated by students t-test for unpaired variates and given as: $^{x}p<0.05$; $^{xx}p<0.02$; $^{xxx}p<0.01$; $^{xxxx}p<0.002$; $^{xxxxx}p<0.001$. Treated rats were orally administered with 66mg/Kg body weight of α-amyrin palmitate every 48 hrs from days 32 to 40 postadjuvant and assessed on day 50.

TABLE 6 Blood cell status of adjuvant arthritic rats treated with α-amyrin palmitate.

	Control	Untreated arthritic	Treated arthritic
Total WBC (10^6/L)	6713 ±810	8088 ± 1508	6225 ± 1369
Granulocytes (10^6/L)	668 ± 72	2287 ± 592 ^{xx}	1813 ± 580
Lymphocytes (10^6/L)	5866 ±721	5581 ±1032	4228 ± 796
Monocytes (10^6/L)	179 ± 42	220 ± 45	183 ± 39
Total Neutrophils (%WBC)	9.1 ± 0.8	26.8 ± 4.2^{xxxx}	24.5 ± 2.6^{xxxxx}
Segmented Neutrophils (%WBC)	7.6 ± 0.8	21.9 ± 3.9^{xxx}	17.5 ± 1.8^{xxxxx}
Banded Neutrophils (%WBC)	1.5 ± 0.3	4.9 ± 1.2^{xx}	7.0 ± 1.2^{xxxx}
Total RBC (10^{12}/L)	8.36 ±0.06	7.97 ± 0.15^{x}	8.22 ± 0.18
Reticulocytes (%RBC)	11.00 ±1.10(6)	14.00 ± 1.15	16.38 ± 1.24^{x}
Hb (g/dL)	16.4 ±0.10	14.6 ± 0.3^{xxxxx}	15.1 ± 0.4^{xxx}
Hct (%)	45.2 ± 0.2	41.5 ± 1.1^{xxx}	42.9 ± 1.2
MCV (fL)	54.0 ± 0.4	51.9 ± 0.8^{x}	52.1 ± 0.4^{xxx}
MCH (pg)	19.6 ± 0.2	18.3 ± 0.2^{xxxxx}	18.3 ± 0.1^{xxxxx}
MCHC (g/dL)	36.2 ± 0.2	35.2 ± 0.3^{xx}	35.2 ± 0.2^{xxx}
Platelets (10^9/L)	786 ± 23	826 ± 41	816 ± 43

See legend for Table 5

TABLE 7 Organ status of adjuvant arthritic rats treated with α-amyrin palmitate.

	Control	Untreated arthritic	Treated arthritic
Liver weight (g%)	3.47 ± 0.06	3.53 ± 0.09	3.64 ± 0.14
Kidney weight (g%)	0.66 ± 0.01	0.70 ± 0.02	0.71 ± 0.03
Spleen weight (g%)	0.20 ± 0.001	0.27 ± 0.01^{xxxx}	0.26 ± 0.02^{xx}
SGOT (U/L)	141 ± 13	128 ± 15	183 ± 21
SGPT (U/L)	93 ± 3	95 ± 4	98 ± 6
Billirubin (μmol/L)	4.63 ± 0.46	3.88 ± 0.48	5.50 ± 0.85
Glucose (mmol/L)	7.88 ± 0.50	7.36 ± 0.68	8.46 ± 0.73
Urea (mmol/L)	6.61 ± 0.36	5.71 ± 0.26	5.71 ± 0.17^{x}
Creatinine (μmol/L)	35 ± 2	33 ± 1	32 ± 1

See legend for Table 5.

EXAMPLE 4

Effect of α-amyrin and α-amyrin esters on 5-lipoxygenase activity in human neutrophils.

A. Materials and Methods.

Drug preparation.

α-amyrin was purchased from ICN (USA) and the chlorides of lauric, myristic, palmitic, stearic, oleic and linoleic acids were purchased from Sigma (USA). In the preparation of lipid esters, α-amyrin was dissolved in dichloromethane (DCM) and equivalent moles of pyridine and the lipid chloride were added under dry nitrogen atmosphere. The DCM extract was dried over anhydrous sodium sulphate, concentrated to the oil and the oil applied to a SiO_2

preparative plate run on petroleum ether spirit. The appropriate band was then separated and identified by NMR spectroscopic analysis.

Lipoxygenase test method.

The method described elsewhere (Cleland *et al.*, 1990) was based on calcium ionophore (A23187) - induced synthesis of 5-lipoxygenase products (Table 8) by human neutrophils in the presence of exogenous arachidonic acid. 1 ml of $2.7x10^6$ neutrophils was used. Measurements were done in quadruplicate on 1:100 dilution of approximately 3.6 mg/ml stock solution of test compound. Data was analysed by multiple t-test in which unpaired Student's t-test was used to compare the test compounds with the single ethanol control (Sinclair, 1988). A $P<0.05$ was considered significant.

B. Results and Discussion.

The 5-lipoxygenase enzyme in human neutrophils catalyses the oxidation of membrane arachidonic acid to 5-HPETE from which 5-HETE and the unstable precursor of the leukotrienes, LTA_4, are formed (Lewis *et al.*, 1990; Samuelsson and Funk, 1989). Inhibition of 5-lipoxygenase would be expected to inhibit all the 5-lipoxygenase products measured here. In the present study, inhibition of 5-HETE synthesis was observed to varying degrees without inhibition of LTB_4 synthesis. As shown in Table 8, α-amyrin and α-amyrin linoleate showed the greatest and most significant reductions in 5-HETE synthesis (-27% and -63% respectively). And while all the other triterpenes tended to increase the synthesis of the leukotrienes, α-amyrin and α-amyrin linoleate reduced the levels of isomer I (-48% and -25% respectively; latter only apparently, $P<0.1$) and isomer II (-39% and -37% respectively) but had no effect on LTB_4.

In rheumatoid arthritis, LTB_4 and 5-HETE increase in the synovial fluid and synovial tissue (Klickstein *et al.*, 1980; Davidson *et al.*, 1982; Davidson *et al.*, 1983) but the relative importance of the two lipoxygenase products is unknown. The knowledge that intra-articular injection of corticosteroids reduce LTB_4 but not 5-HETE (Klickstein *et al.*, 1980) and that LTB_4 is the most

significant proinflammatory substance among the 5-lipoxygenase products (Goetzl and Sun, 1979; Palmblad *et al.*, 1981; Ford-Hutchinson *et al.*, 1981; Goetzl *et al.*, 1980) would suggest that inhibition of LTB_4 is more important in the curtailment of chronic inflammation. Evidence is accumulating however that 5-HETE primes neutrophils for the expression of cellular functions associated with inflammation such as by augmenting cytosolic calcium and mobilising protein kinase C from cytosol to membrane (Lewis *et al.*, 1990; Samuelsson and Funk, 1989). Therefore, it is suggested that inhibition of 5-HETE synthesis may be relevant in antiarthritic therapy.

TABLE 8 Effect of α-amyrin esters on neutrophil synthesis of 5-lipoxygenase products.

	ng/10^6 neutrophils ± S.E.M.			
	5-HETE	Isomer I	Isomer II	LTB_4
Ethanol Control	278.3 ± 28.2	13.4 ± 1.3	13.6 ± 1.4	9.4 ± 0.5
A	202.1 ± 7.5^{x}	7.0 ± 0.6^{xxx}	8.3 ± 0.7^{xx}	9.8 ± 0.4
LU	243.0 ± 6.3	20.4 ± 0.5^{xxx}	19.3 ± 0.5^{xxx}	11.6 ± 0.1^{xxx}
MA	251.1 ± 14.9	15.3 ± 1.9	14.2 ± 1.9	10.1 ± 0.7
PA	261.1 ± 2.6	23.2 ± 0.5^{xxxxx}	22.5 ± 0.9^{xxxx}	15.0 ± 0.4^{xxxxx}
SA	234.6 ± 3.2	15.9 ± 0.6	15.1 ± 0.8	11.4 ± 0.4^{xx}
OA	253.4 ± 4.9	17.0 ± 0.3^{x}	15.4 ± 0.4	11.0 ± 0.2^{xx}
LA	103.2 ± 4.9^{xxxxx}	10.1 ± 0.8	8.5 ± 0.6^{xx}	10.4 ± 0.4

Values are mean ± S.E.M. of 4 experiments.
Significance of difference from control was calculated by the Student t-test for unpaired variates and given as: $^{x}P<0.05$; $^{xx}P<0.02$; $^{xxx}P<0.01$; $^{xxxx}P<0.002$; $^{xxxxx}P<0.001$.

A, α-amyrin; LU, α-amyrin laurate; MA, α-amyrin myristate; PA, α-amyrin palmitate; SA, α-amyrin stearate; OA, α-amyrin oleate; LA, α-amyrin linoleate; 5-HETE = 5(S)-Hydroxy-(E,Z,Z,Z)-6,8,11,14-eicosat tra noic acid; LTB4 = 5(S,12R)-5,12-dihydroxy-(Z,E,E,Z)-6,8,10,14-eicosatetraenoic acid; Isomer I = 6-trans-leukotriene B_4 or (5S,12R)-5,12-dihydroxy-(E,E,E,Z)-6,8,10,14-eic satetraenoic acid. Isomer II = 6-trans-12-epi-leukotriene B_4 or (5S,12R)-5,12-dihydr xy-(E,E,E,Z)-6,8,10,14-eicosatetraenoic acid.

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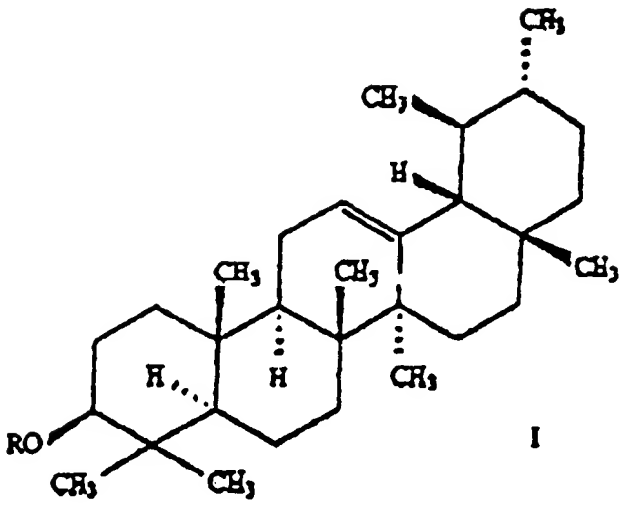
CLAIMS:

1. A compound of the general formula I:



I

wherein R represents an acyl group derived from a long chain fatty acid.

2. A compound according to claim 1, in substantially pure form.

3. A compound according to claim 1 or claim 2, wherein the acyl group is derived from a saturated or unsaturated fatty acid having 10 or more carbon atoms.

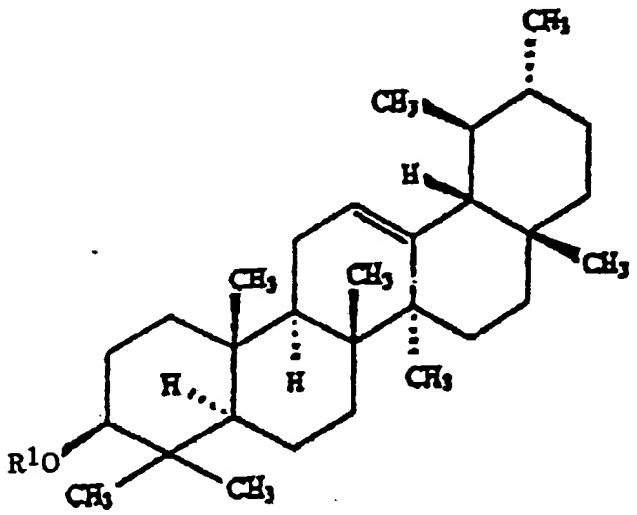
4. A compound according to claim 3, wherein the acyl group is derived from capric, lauric, myristic, palmitic, stearic, arachidonic, myristoleic, palmitoleic, oleic, linoleic, linolenic or arachiodonic acid.

5. α-amyrin palmitate.

6. A process for the production of a compound according to claim 1, which comprises esterification of α-amyrin with a long chain fatty acid or an acid halide or other derivative thereof.

7. A process according to claim 6, wherein α-amyrin is esterified with palmitic acid or palmitoyl chloride.

8. A method of antiinflammatory, and more particularly antiarthritic, treatment of a human or animal patient, which comprises administering to said patient an effective amount of a compound of the general formula II in substantially pure form:



II

wherein R^1 represents hydrogen or an acyl group derived from a long chain fatty acid.

9. A method according to claim 8 wherein in the compound of the general formula II, R^1 represents an acyl group derived from a saturated or unsaturated acid having 10 or more carbon atoms.

10. A method according to claim 9 wherein in the compound of the general formula II, R^1 represents an acyl group derived from capric, lauric, myristic, palmitic, stearic, arachidonic, myristoleic, palmitoleic, oleic, linoleic, linolenic or arachiodonic acid.

11. A method according to claim 8 wherein the compound of the general formula II α-amyrin or α-amyrin palmitate.

12. The use of a compound of the general formula II as defined in any one of claims 8 to 11 in substantially pure form in the manufacture of a medicament for antiinflammatory, and more particularly antiarthritic, use.

13. A pharmaceutical composition for antiinflammatory, and more particularly antiarthritic, use comprising a compound of the general formula II as defined in any one of claims 8 to 11 in substantially pure form, together with a pharmaceutically acceptable carrier or diluent.

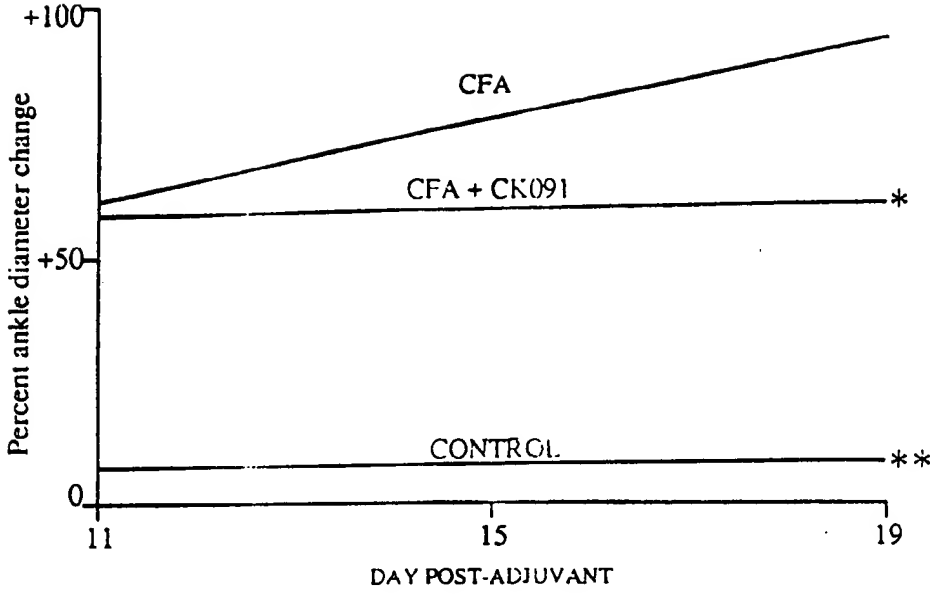

+100
+50
0
Percent ankle diameter change
CFA
CFA + CK091
CONTROL
*
**
11
15
19
DAY POST-ADJUVANT

FIGURE 1

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[5] C07J 063/00, A61K 031/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07J 063/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

AU: IPC: C07J 063/00

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)

Chemical Abstracts: STN Database: Structure searching of compound of formula I.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X P,Y	KWEIFIO-OKAI, G., "Antiinflammatory activity of a Ghanaian antiarthritic herbal preparation:1",Journal of Ethnopharmacology 33 (1991) 263-267	1-5 8-13
P,Y P,A	Kweifio-Okai, G., "Antiinflammatory activity of a Ghanaian antiarthritic herbal preparation: II", Journal of Ethnopharmacology 33 (1991) 129-133	8-13 1-5
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[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 1993 (05.02.93)	16 FEB 1993 (16.02.93)

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate f the relevant passages	Relevant to Claim No.
X	GÜLZ, P-G et al, "Epicuticular leaf waxes of citrus halimii stone" J. Agric. Food Chem. 1987, 35, 716-720	1-5
X	GÜLZ, P-G et al, "Epicuticular wax of euphorbia aphylla Brouss ex Willd, Euphorbiaceae", Z. Naturforsch 43c 19-23 (1988)	1,3,4
X	HEMMERS, H. et al, "Tetra and pentacyclic triterpenoids from epicuticular wax of euphorbia cyparissias L., Eurphorbiaceae", Z Naturforsch 44c 563-567 (1989)	1,3,4
X	HEMMERS, H. et al, "Triterpenoids in epicuticular waxes of three European euphorbia species", Z. Naturforsch 43c 799-805 (1988).	1,3,4
X	NIEMANN, G.J. et al, "Biosynthesis and accumulation of triterpenoids in epidermis and mesophyll of ilex aquifolium leaves in relation to the age of the leaf", J. Plant Physiol. Vol. 129, 395-405 (1987)	1,3,4,5
X	ERCIYAS, E. et al, "Alpha-amyrin tetratriacontanoate, a new triterpene from scolymus hispanicus L. ". Die Pharmazie V44(8) 1989 page 580.	1,3
X A	AGNIHOTRI, V.K. et al "Constituents from the seeds of cordia obliqua as potential anti-inflammatory agents" Indian J. Pharmaceutical Sciences V49 No. 62, 1987 pages 66-69.	8,11,12 9,10
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